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AeiA, an Atg8-interacting protein in *Aspergillus oryzae*, promotes peroxisome degradation by pexophagy

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ABSTRACT

There are two types of autophagy, non-selective (bulk) autophagy, in which substrates are randomly incorporated into autophagosomes, and selective autophagy, in which substrates are specifically targeted. In filamentous fungi, the molecular mechanism underlying selective autophagy remains largely unknown. Recently we identified a novel protein, AoAtg8-interacting protein A (AeiA), in the filamentous fungus *Aspergillus oryzae*. AeiA was localized to peroxisomes and autophagosomal intermediates, such as phagophore assembly site (PAS) and the phagophore. Moreover, pexophagy flux was reduced in AeiA deletants. Taken together, AeiA is a novel selective autophagy-related protein that contributes to pexophagy in *A. oryzae*. Our findings provide insight into the molecular mechanisms of selective autophagy including pexophagy in filamentous fungi.

Abbreviations: AIM, Atg8-family interacting motifs; Atg8, autophagy-related 8; EGFP, enhanced green fluorescent protein; GABARAP, Gamma aminobutyric acid A receptor associated protein; LC3, Microtubule-associated protein light chain 3; MTS, microbody targeting signal; PD, potato dextrose.

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KEYWORDS Atg8; peroxisome; pexophagy; filamentous fungi; *Aspergillus oryzae*

The filamentous fungus *Aspergillus oryzae* is an important microorganism in the Japanese fermentation industry and has recently been used as a host for both homologous and heterologous protein production. In addition, *A. oryzae* has been used as a model organism for studying autophagy in filamentous fungi, and unique autophagic processes in this organism have been reported, including the degradation of peroxisomes, mitochondria and even nuclei via autophagy, suggesting that these organelles are targets of selective autophagy.

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Recent studies in yeast and mammalian cells have revealed the mechanism of how specific organelles are recognized by the so-called selective autophagy receptors. Each organelle has at least one selective autophagy receptor that serves as a degradation marker for its specific turnover by autophagy. These receptors bind to the Atg8 pool (LC3 or GABARAP in mammals) on the phagophore membrane and guide its elongation and closure along the target organelle. This mechanism provides selective autophagy with specificity.

In filamentous fungi such as *A. oryzae*, however the molecular mechanism of selective autophagy is largely unknown. This is due to the low homology of primary amino acid sequences with those of selective autophagy-related proteins in other species, making it difficult to search for candidate proteins from genome databases. We therefore decided to purify proteins that interact with *A. oryzae* Atg8 (AoAtg8) and screen for specific binding partners, including selective autophagy receptors. In a recent study, we reported the identification of AeiA (AoAtg8-interacting protein A), which has three Atg8-family interacting motifs (AIMs) and a microbody targeting signal (MTS) in its C-terminus [1].

First, we examined the subcellular localization of AeiA. When the co-localization of AeiA with AoAtg8 was found that some of the dot-like structures formed by AeiA co-localized with AoAtg8-positive puncta, indicating that AeiA can localize to the PAS [1]. In addition, AeiA was also localized in a large cup-shaped isolation membrane marked with EGFP-AoAtg8 under carbon source starvation conditions, strongly suggesting that AeiA is an autophagy-related protein.

Since AeiA harbored a MTS, we verified the localization of AeiA with peroxisomes, which are a type of microbody. Part of AeiA was localized to small peroxisomes under normal growth conditions. When shifted to carbon source starvation conditions, AeiA localized to the developed large peroxisomes. If AeiA interacts with AoAtg8 and guide pexophagy, it should be present on the membrane of peroxisomes. However, AeiA has no transmembrane domains. Thus, we speculate that AeiA interacts with other proteins such as Pex5, which is a PTS receptor on the peroxisome surface, to recruit AoAtg8 (Figure 1), but this scenario still needs to be investigated.

The localization of AeiA to autophagic intermediates suggested that AeiA might be involved in non-selective autophagy. Defects in non-selective, bulk autophagy in *A. oryzae* cause a disturbance in the formation of aerial hyphae and conidia. Therefore, to elucidate the function of AeiA in *A. oryzae*, we constructed the *aeiA* disruptant *ΔaeiA*. The *ΔaeiA* strain was inoculated on PD agar medium and cultured for 5 days, but there was no difference in aerial hyphae formation and conidiation compared to the control. This result suggested that the disruption of *aeiA* does not affect bulk autophagy. Furthermore, fluorescence microscopy analysis of EGFP-AoAtg8 expressed

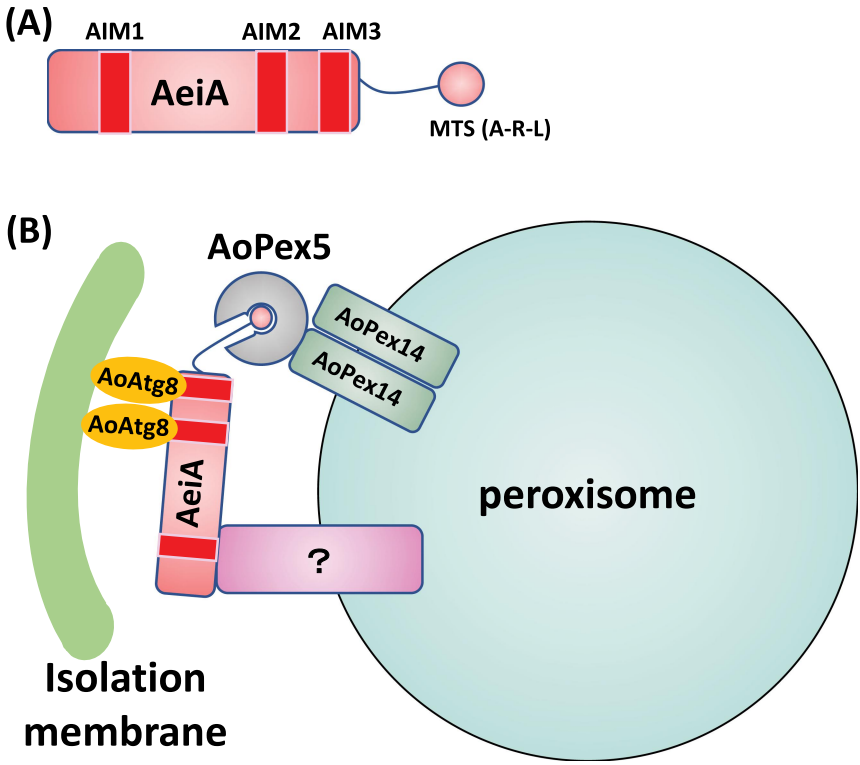

Figure 1. A speculative model for the action of AeiA in pexophagy (A) Schematic representation of AeiA structures. AeiA interacts with AoAtg8 via AIM2 and AIM3 [1]. MTS consists of the amino acid sequence A-R-L. (B) Putative role of AeiA in pexophagy. AeiA associates to peroxisomes by binding via its MTS the PTS receptor AoPex5, which form a complex with the peroxisomal membrane protein AoPex14. In addition, AeiA might be recognized by an unknown protein present on peroxisomal membrane, because AeiA appeared to be transported to the lumen of peroxisomes [1]. Under pexophagy inducing condition such as growth in oleic acid-containing medium, AeiA interacts with AoAtg8 pool present on the phagophore to promote sequestration of peroxisomes within autophagosomes.

in the *ΔaeiA* strain showed that EGFP-AoAtg8 fluorescence in the vacuoles under carbon or nitrogen source starvation conditions. In addition, examination of EGFP-AoAtg8 processing by western blot showed that free EGFP appears under starvation conditions. These results indicated that AeiA may be part of a selective autophagy pathway.

Thus, since AeiA also localized to peroxisomes, we next examined the effect of AeiA on pexophagy. We quantified the number of peroxisomes remaining in the cells by microscopy under pexophagy inducing conditions, and found that the number of peroxisomes in the *ΔaeiA* strain was higher in comparison to the wild type. Pexophagy was also

evaluated by the AoPex11-1-EGFP processing assay, in which EGFP was fused to peroxisome localized AoPex11-1, and showed a reduced pexophagy flux in the *ΔaeiA* strain, revealing that AeiA is a novel protein that is specifically associated with pexophagy.

AeiA has no homologues in yeast or mammals. Therefore, AeiA is a novel and unique protein in filamentous fungi especially *Aspergillus* species that is specifically involved in pexophagy. One reason behind the fact that a few selective autophagy-related proteins in filamentous fungi have been discovered so far is due to the low conservation of their primary amino acid sequences within eukaryotes. Thus, we speculate that the molecular mechanisms that generate the degradation of specificity substrates by selective autophagy have been acquired by convergent evolution. Further functional analyses of AoAtg8-interacting proteins are required to identify novel selective autophagy-related proteins and to elucidate the molecular mechanisms of selective autophagy in filamentous fungi, including selective degradation of whole nuclei by nucleophagy, which is unique to *A. oryzae*.

Disclosure statement

No potential conflict of interest was reported by the authors.

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